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Red wine and polyphenols display antimicrobial effects in an oral bacteria biofilm model

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Running head: Oral biofilm antimicrobial effects of red wine

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Abstract

The antimicrobial effects of red wine and its inherent components on oral microbiota have been studied by using a 5-species biofilm model of the supragingival plaque that includes *Actinomyces oris*, *Fusobacterium nucleatum*, *Streptococcus oralis*, *Streptococcus mutans* and *Veillonella dispar*.

Microbiological analysis (CFU counting and confocal laser scanning microscopy) of the biofilms after the application of different test solutions (red wine, dealcoholized wine, red wine extract, grape seed extract and inactive dry yeast extracts) showed that the grape seed extract solution was the most effective, exhibiting high activity against *F. nucleatum*, *S. oralis* and *A. oris*. Additionally, both red wine and dealcoholized red wine had an antimicrobial effect against *F. nucleatum* and *S. oralis*.

Additional experiments were carried out to determine any possible phenolic metabolism during formation of the bacterial biofilm. Flavan-3-ol precursors such as (+)-catechin and procyanidin B2 suffered an almost complete and early degradation when incubating biofilms with the red wine extract but no degradation was observed after incubation with the grape seed extract, probably due both to its higher concentration and antimicrobial effects. To our knowledge, this is the first study of antimicrobial properties of wine in an oral biofilm model.

Introduction

The oral cavity is an enormously complex habitat with several hundred commensal microbial species colonizing it, and furthermore it is unique in the human body in possessing non-shedding surfaces, the teeth, allowing microorganisms to adhere to the surface of teeth for long periods of time, embedded in a self-produced matrix of extracellular polymeric substances (Abee *et al.*, 2011), and thus leading to extensive biofilm formation, dental plaque (Marsh, 2003), which is more resistant than planktonic cells to mechanical stress or antibiotic treatment (Roberts *et al.*, 2010). The microorganisms of dental plaque live with one another in a commensal or mutualistic symbiotic relationship, allowing a mixture of aerobic and anaerobic bacteria to live in the same environment. Some of these oral bacteria such as streptococci or lactobacilli are able to produce high levels of organic acids following fermentation of dietary sugars. Acids released from dental plaque lead to demineralization of the tooth surface and consequently to dental caries, periodontal disease or tooth loss (Hardie, 1992), which are the most prevalent oral diseases in humans, affecting up to 60–90% of the world population (da Silva *et al.*, 2013).

Even using mechanical removal, dental biofilms cannot be eliminated completely. Antimicrobial agents are complementarily used to control dental plaque (Furiga *et al.*, 2008; Marsh *et al.*, 2010; Kamonpatana *et al.*, 2012). Until now, several substances have been tested for the control of oral biofilms, including essential oils, amine fluoride, triclosan, etc., but one of the most widely used and effective antibiofilm agents is chlorhexidine (Corbin *et al.*, 2011). However, chlorhexidine has been associated with some secondary effects, namely the reduction of human taste perception and the pigmentation of oral tissues, which limits its application. Therefore, the search for new antimicrobials has arisen, and natural products are preferable due to the lack of secondary effects and therefore, the potential for long-term usage in the oral cavity.

The inherent matrix of the biofilm, such as extracellular polymeric substances that reduce penetration of antimicrobial agents and the presence of persister cells surviving at low metabolic rates, contributes to the widely described phenomenon of reduced sensitivity to antimicrobial agents (Hoyle & Costerton, 1991). Because of this, biofilm models including bacteria and fungi from different species have proven both useful and reliable and reliability in

predicting *in vivo* efficacy of antimicrobials. In this sense, most experimental models for short-term studies involve a solid surface for the adhesion of bacteria (Guggenheim *et al.*, 2004).

Although there is substantial literature reporting the antimicrobial properties of phenolic compounds or polyphenols against bacteria isolates (Jayaprakasha *et al.,* 2003; Ozkan *et al.*, 2004; Cueva *et al.*, 2010), information about their effect on oral pathogens is still scarce (Requena *et al.*, 2010). Studies carried out with tea and cranberry polyphenols have shown an inhibitory effect on biofilm formation by oral pathogens such as *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sobrinus* and *Porphyromonas gingivalis* (Bodet *et al.*, 2008). Grapes and wines are good dietary sources of polyphenolic compounds, including hydroxybenzoic and hydroxycinnamic acids, phenolic alcohols, flavan-3-ol monomers, oligomeric and polymeric procyanidins, flavonols, stilbenes and anthocyanins (only present in red varieties) (Monagas *et al.*, 2003). Recently, it has been found that wine and grape phenolic extracts, as well as pomace phenolic extracts, were able to inhibit the growth of different *Streptococcus* spp. strains associated with dental caries (Thimothe *et al.*, 2007; Furiga *et al.*, 2009).

On the other hand, interactions between wine phenolics and oral microbiota can also include a possible bacterial catabolism of wine phenolics into less complex phenolic metabolite structures as seems to happen with flavonol glycosides (Requena *et al.*, 2010). With regards to anthocyanins, their degradation in human saliva at 37 ºC has been described, being structure-dependent, largely mediated by oral microbiota, and partially suppressed after oral rinsing with antibacterial chlorhexidine (Kamonpatana *et al.*, 2012).

With the final aim of seeking natural products that could be used in oral hygiene and to ascertain interactions between wine components and oral microbiota, in this study the antimicrobial effects of red wine and dealcoholized red wine were investigated using a biofilm model of the supragingival plaque that integrates five bacteria species commonly associated with oral disease. A wine phenolic extract (Provinols™), especially rich in anthocyanins, was also tested using the same model, and in both the absence and presence of other enological extracts from grape seeds (Vitaflavan®) and yeast (inactive dry yeast, IDY). Additional experiments were carried out to determine any possible phenolic metabolism during the formation of the bacterial biofilm.

Materials and methods

Red wines

The red wine used in this study was a young red wine (var. Pinot Noir, vintage 2010), kindly provided by Bodegas Miguel Torres S.A. (Catalonia, Spain). The wine was elaborated following the winery's own winemaking procedures and was selected because of its relatively high phenolic content: total polyphenols = 1758 mg of gallic acid equivalents/L, total anthocyanins = 447 mg of malvidin-3-glucoside/L, and total catechins = 1612 mg of (+)-catechin/L. Main individual phenolic compounds found in this wine included anthocyanins, flavan-3-ols, flavonols, alcohols, stilbenes and hydroxycinnamic acids (Muñoz-González *et al.*, 2013) (Table 1).

For the preparation of dealcoholized red wine, ethanol was removed using a rotary evaporator and then distilled water was added until the original volume was reached.

Enological extracts

A wine extract, Provinols™, was kindly supplied by Safic-Alcan Especialidades S.A.U. (Barcelona, Spain). A grape seed extract, Vitaflavan® was kindly provided by Dr. Piriou (Les Dérivés Resiniques & Terpéniques S.A., France). The total phenolic content of the extracts was 474 mg of gallic acid equivalents/g for Provinols™ and 629 mg of gallic acid equivalents/g for Vitaflavan®. The main phenolic compounds identified in both extracts are reported in Table 1. Also, two inactive dry yeast (IDY) commercial preparations (*Saccharomyces cerevisiae*), IDY 1 and IDY 2, rich in mannoproteins, aminoacids and peptides, respectively, were kindly provided by Lallemand S.A. (Blagnac, France) and Agrovin S.A. (Alcázar de San Juan, Ciudad Real, Spain).

The wine extract was dissolved in distilled water containing 2.5% DMSO (v/v), at a concentration of 1.6 g/L. The wine extract solution was fortified in grape seed polyphenols by adding 2.5 g of grape seed extract to 100 mL of the wine solution. Also, the wine extract solution was enriched in wine matrix components (mainly polysaccharides and nitrogen

compounds) by adding the IDY preparations to the wine extract solution at a final concentration of 0.4 g/L.

Bacterial strains and culture (growth) conditions

Actinomyces oris OMZ 745*, Fusobacterium nucleatum* OMZ 598*, Streptococcus oralis* OMZ 607*, Streptococcus mutans* UA159 (OMZ 918) and *Veillonella dispar* ATCC 17748^T (OMZ 493) were obtained from the culture collection of the Institute of Oral Biology, University of Zürich. Prior to the experiment, pre-cultures were prepared by transferring the strains on Columbia Blood Agar plates and incubating them for 96 h at 37 ºC under anaerobic conditions. After this time, the strains were transferred from the Columbia Blood Agar plates to broth cultures (1 x 9 ml FUM in Sørensen's buffer + 0.3 % glucose) (OMZ 493: + 1% sodium lactate) and incubated overnight at 37 ºC. After incubation, 200 µL of bacteria from each working culture were individually inoculated in 5 mL of fresh FUM media in Sørensen's buffer and incubated at 37 ºC anaerobically (7 hours maximum). In order to obtain an inoculum containing cultures in the exponential growth phase of approximately 10^7 CFU/mL, a microbial suspension with equal volumes and densities of each strain was prepared.

Saliva processing

Saliva was collected and processed according to the protocol of Guggenheim *et al.* (2001). Briefly, whole unstimulated saliva was collected from volunteers for 1 h each morning, over several days, at least 1.5 h after eating, drinking, or teeth cleaning. Saliva samples were collected in sterile 50 mL polypropylene tubes, chilled in an ice bath or frozen at −20 °C. After 500 mL saliva had been collected, it was pooled and centrifuged (30 min, 4 °C, 27,000 × *g*); the supernatant was pasteurized (60 °C, 30 min) and re-centrifuged in sterile tubes. The resulting supernatant was stored in sterile 50 mL polypropylene tubes at −80 °C. The efficiency of the process was assessed by plating the processed saliva samples onto CBA agar; after 72 h at 37 °C, no CFUs were observed on the incubated plates. A sterile 1:1 dilution in H_2O+25% physiological NaCl was used for the biofilm formation and throughout the experimentation.

In vitro biofilm experiments

Figure 1 shows a sequence chart regarding the biofilm formation prior to assays for determining changes in the microbial population of the biofilm and for assessing phenolic metabolism in the biofilm.

Biofilm formation

Biofilms were grown using the slightly modified protocol described by Guggenheim *et al.* (2001) and Thurnheer *et al.* (2006). In brief, the 5-species biofilms were grown in 24-well polystyrene cell-culture plates on hydroxyapatite (HA) discs of 9mm Ø (Clarkson Chromatography Products, South Williamsport, USA) previously preconditioned in 800 µL of whole unstimulated pooled saliva (as described in the previous section) during 4 h at room temperature, with shaking (95 rpm) in order to promote pellicle formation. To initiate the biofilm formation, the discs were covered for 45 minutes with 1.6 mL of a mixture comprising 30% saliva, 70% modified fluid universal medium (mFUM) and 200 µL of the bacterial inoculum described above. mFUM corresponds to a well-established tryptone yeast-based broth medium designated as FUM (Gmür & Guggenheim, 1983) and modified by supplementing 67 m M Sørensen's buffer (final pH 7.2). The carbohydrate concentration in mFUM was 0.3% (w/v) and consisted of glucose for the first 16 h and from then on of a 1:1 (w/w) mixture of glucose and sucrose.

After this first incubation, discs were subjected to three consecutive 1 min dip-washes in 2 ml 0.9% NaCl to remove growth medium and free-floating cells but not microorganisms adhering firmly to the HA discs. Then, they were incubated anaerobically for 16.5 h at 37 °C in preconditioned and processed saliva to form the biofilm (Figure 1).

Assay for determining changes in the microbial population of the biofilm

Once the biofilm was formed, discs were maintained in a 24-well plate with preconditioned and processed saliva in anaerobic conditions for 7 days. Twice a day, and with 7 hours of difference in between, discs were "fed" by immersing them into a preconditioned fresh growth medium (30% saliva, 70% mFUM (v/v) containing 0.15% glucose and 0.15 % sucrose) for 45

minutes, at 37 ºC, under anaerobic conditions, After each "feeding", discs were dipped in the different test solutions (1 mL) for 2 minutes and while being gently shaken by hand. After this time, the discs were dipped once in the preconditioned-processed saliva in order to clean any remains of the test solutions. Immediately after, discs were returned to the "old" 24-well plate with preconditioned and processed saliva and incubated anaerobically until the next "feeding" (Figure 1). After 7 days, biofilms were either stained for confocal laser scanning microscopy (see below) or harvested, at room temperature, in 1 mL of 0.9% NaCl by scratching with a special odontological instrument. Cell viability was tested using a Live/Dead BacLight Viability Kit (Molecular Probes Inc.) The total CFU, streptococci and all taxa were assessed by anaerobic culture (37 ºC) using selective (Mitis Salivarious for *Streptococcus oralis* and *Streptococcus mutans*; Fastidious Anaerobe Agar for *Fusobacterium nucleatum*) and non-selective media (Columbia Blood Agar for *Actinomyces oris*, *Veillonella dispar* and total CFU) and colonies were counted.

Distilled water was used as the negative antimicrobial control, and 0.2% chlorhexidine-gluconate solution (Sigma-Aldrich, Steinheim, Germany) in water was the positive antimicrobial control. In order to discard a possible antimicrobial effect of the alcohol12% ethanol in water was also tested. For both, test solutions and controls, experiments were carried out in triplicate.

Assay for assessing phenolic metabolism in the biofilm

After initiating biofilm formation as described above, the 70:30 saliva:mFUM media was enriched with the wine extract (1.6 g/L) in the absence of the presence of grape seed extract (10 g/L) and added into the wells containing the discs (Figure 1). Then, plates were incubated at 37 ºC under anaerobic conditions and aliquots of enriched media were taken at 0, 2, 4, 6, 8 and 24 hours.

Analysis of wine compounds and bacterial/microbial metabolites

Phenolic compounds were analyzed using an UPLC-ESI-MS/MS following a previously reported method (Muñoz-González *et al.*, 2013). The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with a binary pump, an autosampler

thermostatted at 10 ºC, and a heated column compartment (40 ºC). The column employed was a BEH-C18, 2.1 x 100 mm and 1.7 µm particle size from Waters (Milford, MA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 mL/min and injection volume was 2 µL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 ºC; desolvation temperature, 400 ºC; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids and valerolactones) were previously reported (Jiménez-Girón *et al.*, 2013). Data acquisition and processing was realized with MassLynx 4.1 software.

Staining of biofilms and confocal laser scanning microscopy (CLSM)

For CLSM, treated as well as untreated biofilms were stained using the LIVE/DEAD BacLight bacterial viability assay (Invitrogen, Zug, Switzerland) according to the instructions of the manufacturer. After 20 min staining, excess dye was gently aspirated from the discs without touching the biofilms. They were embedded upside-down in 20 µl of Mowiol (Thurnheer *et al.*, 2003) and stored at room temperature in the dark for at least 6 h prior to microscopic examination.

Stained biofilms were examined by CLSM at randomly selected positions using a Leica TCS SP5 (Leica Microsystems, Heidelberg GmbH, Germany) with a x20/0.8 numerical aperture (NA) and x63/1.4 NA oil immersion objective lens in conjunction with 488-nm laser excitation

and 530-nm emission filters for Syto 9 (live stain), and 561-nm laser excitation and 640-nm emission filters for propidium iodide (dead stain). Image acquisition was done in 8-line average mode and the data were processed using Imaris 7.2.2 (Bitplane AG, Zurich, Switzerland).

Statistical analysis

Means and standard deviations were calculated using Microsoft Excel 2007. Statistical analyses were performed through Statistica©. To compare the antimicrobial effects of the different treatments with the control (water), the Dunnett test was applied. Graphs were performed with Microsoft Excel 2007.

Results and discussion

Antimicrobial properties of wine and dealcoholized red wine on the biofilm

The effects of a red wine and the same wine without ethanol on a biofilm model comprised of five representative species commonly encountered in supragingival plaque, including Gram-positive (*A. oris, S. mutans, S. oralis*) as well as Gram-negative (*F. nucleatum, V. dispar*) bacteria (Guggenheim *et al.*, 2001), were investigated. Among these bacteria were the so-called early colonizers, *A. oris*, *S. oralis* and *V. dispar*, and late colonizers, *S. mutans* and *F. nucleatum*, the latter also designated as a bridging organism due to its capability to co-aggregate with a wide range of early and late colonizers (Kolenbrander *et al.*, 2006). When discs were dipped into both red wine and dealcoholized red wine, some decrease in cell viability of the whole biofilm was visually estimated (Figure 2C) in comparison to the control (Figure 2A). CFU values for the five bacteria comprising the biofilm indicated an important reduction in *F. nucleatum* and *S. oralis* population when applying red wine and dealcoholized red wine to the biofilm, in comparison to the negative control (distilled water) (Table 2). The Dunnett test confirmed significant differences in the population of these two strains after the treatment with wine and dealcoholized wine. Generally, wines contain between 10–12% of ethanol, which have antimicrobial properties. To understand the action mechanism of red wines in more depth, the effects of ethanol of the bacteria biofilm were investigated. The treatment with 12% ethanol resulted in a significant decrease in the population of *F. nucleatum*

283 (Table 2). However, since treatments of the biofilm with both wine and dealcoholized wine
284 inhibited *F. nucleatum* growth, it was likely that other wine components – apart from ethanol –
285 had antimicrobial properties against this bacteria species. As expected, all the strains were
286 eradicated after the treatment with the positive control (0.2% chlorexidine-gluconate solution)
287 (Table 2).

288 In an intervention study with 75 volunteers, Signoretto *et al.* (2010) analyzed the microbial
289 population of supragingival and subgingival plaque using PCR-DGGE and found that *F.*
290 *nucleatum* was less frequentl in wine drinkers compared with water drinkers. Other authors
291 such as Daglia *et al.* (2007) have also shown antimicrobial properties of dealcoholized wine
292 against oral streptococci. Both studies were consistent with our results in that wine selectively
293 inhibited the growth of *F. nucleatum* and *S. oralis* in the presence of other species such as *S.*
294 *mutans*, *A. oris* and *V .dispar* in an oral biofilm model.

295 Given the antimicrobial effects of wine observed in the first experiment, the next step was to
296 study the influence of some wine-specific components such as polyphenols, including flavan-
297 3-ols, peptides or yeast polysaccharides. For that purpose, a red wine extract solution spiked
298 with different extracts rich in those specific components of wine (grape seed extract rich in
299 flavan-3-ols, and two inactive dry yeasts rich in peptides and mannoproteins, respectively)
300 were used. Table 3 reports the CFU values of the five bacteria species of the tested biofilm
301 after treatments with wine extract and wine extract solution spiked with different extracts
302 (grape seed extract, IDY1 and IDY2). Dunnett's test showed significant differences in *F.*
303 *nucleatum, S. oralis and A. oris* with the application of the wine extract spiked with the grape
304 seed extract rich in flavan-3-ols (Monagas *et al.*, 2003). However, wine extract solutions
305 spiked with IDY1 and IDY2 did not show any effect in the populations of the five-strain
306 biofilm. Notably, a great decrease in the viability of the cell was visually appreciated in the
307 biofilm recovered from the discs that were dipped in the grape seed extract solution (Figure
308 2D). Cueva *et al.* (2012) reported significant inhibition in the growth of some oral streptococci,
309 such as *Streptococcus mutans* and *Streptococcus sobrinus*, when incubating planktonic cultures
310 with flavan-3-ols precursors, (+)-catechin and (-)-epicatechin, in which grape seed extract is
311 particularly rich. Moreover, they showed that extracts from grape seed, especially Vitaflavan®
312 and its oligomeric fraction, exerted higher antimicrobial activity against various oral pathogens
313 than the rest of the extracts tested (red wine extract and grape pomace extract). Similarly,

Rotava *et al.* (2009) and Baydar *et al.* (2006) reported antimicrobial effects of grape seed extracts against pathogenic bacteria such as *S. aureus* and *E.coli*. It has been suggested that the high concentration of flavonoids and their derivatives in grape seeds could be responsible of the antimicrobial activity of grape seed extracts (Anastasiadi *et al.*, 2009). These observations raise the question of how the hydroxyl groups (structure) of flavonoids affect oral bacterial biofilm.

The search for new antimicrobial agents to control the formation of dental plaque requires appropriate screening models that include orally relevant organisms. The model used in this study is not only useful for investigating ecological shifts in plaque composition in response to plaque composition but also for testing the efficacy of antimicrobial agents under conditions of repeated short-term exposure (Guggenheim *et al.*, 2004).

Change in wine phenolic metabolism

Because wine and their polyphenols diminished bacteria population in the oral biofilm, a new assay was performed in order to gain a deeper understanding about microbial metabolism of polyphenols in the tested extracts.

Firstly, the wine extract solution was added to the growth media and the progress of the phenolic metabolism by the five-species biofilm was studied by monitoring changes in the main phenolic compounds present in the wine extract (Table 1), this is to say, flavan-3-ols monomers ((+)-catechin, (-)-epicatechin and (-)-epicatechin 3-O-gallate, dimeric procyanidins (B1, B2, B3, B4, B5, B7, B2-3-O-gallate and B2-3-O-gallate), trimeric procyanidins (C1 and other trimers) and flavonols (quercetin, myricetin, kaempherol, quercetin-3-O glucoside and quercetin-3-O galactoside)). As a brief example, Figure 3 shows the differences in the degradation by the five-strain biofilm of three of the analyzed precursors, (+)-catechin, procyanidin B2 and quercetin, when growing in media enriched with the wine extract solution (1.6 g/L). The UPLC-MS analysis of these three compounds showed high degradation rates, almost completely during the first 2 h of incubation, in the flavan-3-ol precursors, (+)-Catechin and Procyanidin B2, probably because of their low concentration in the media which permitted the bacteria of the biofilm using them as a carbon source. However, no degradation of the precursor quercetin was observed during the incubation period.

To gain further knowledge about the metabolism of grape polyphenols, specifically in flavan-3-ols metabolism, the growing media was enriched by adding, to the red wine extract solution, a concentration of 10 g/L of grape seed extract, which is especially rich in flavan-3-ol precursors. Despite the greater concentration of flavan-3-ol precursors, no degradation of the flavan-3-ol precursors was observed, which could be associated with the high concentration of the studied compounds that, in fact, had an antimicrobial effect on three out of the five bacteria comprising the biofilm, as demonstrated in the antimicrobial assay (previous section). No degradation of precursor quercetin was observed during the incubation period.

The emergence of antibiotic resistance by some oral bacteria biofilm species presents a worldwide problem, and thus novel strategies are required. The use of natural antimicrobials may contribute to controlling the disordered growth of oral microbiota, thus overcoming problems caused by species resistant to conventional antimicrobials (Nascimento *et al*., 2000). To our knowledge, this is the first report on the antimicrobial properties of wine in an oral biofilm model. Our results show that red wines, consumed in moderation, inhibit the growth of some pathogenic species in an oral biofilm model. These findings contribute to existing knowledge about the beneficial effects of red wines (one of the most important products of agriculture and food industries) on human health. Moreover, the promising results concerning grape seed extract, which showed the highest antimicrobial activity, open promising ways towards a natural ingredient in the formulation of oral care products specifically indicated for the prevention of caries, due to its antimicrobial properties.

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462

Table 1. Main phenolic compounds in wine and extracts.

	Wine (mg/L) (Muñoz-González et. al., 2013)	Wine extract (mg/g) (Sánchez-Patán et al., 2012)	Grape seed extract (mg/g) (Sánchez-Patán et al., 2011)
Benzoic acids			
Gallic acid	27.3±0.2	1.06 ± 0.05	9.11 ± 0.01
Protocatechuic acid	3.88±0.01	*n.a.*	*n.a.*
3-O-Methylgallic acid	1.06±0.06	*n.a.*	*n.a.*
4-Hydroxybenzoic acid	0.570±0.008	*n.a.*	*n.a.*
Vanillic acid	1.85±0.03	*n.a.*	*n.a.*
Syringic acid	2.30±0.13	*n.a.*	*n.a.*
Benzoic acid	1.14±0.06	*n.a.*	*n.a.*
Salicylic acid	0.215±0.001	*n.a.*	*n.a.*
Phenols			
Phloroglucinol	0.326±0.030	*n.a.*	*n.a.*
Tyrosol	31.4±1.4	18.9 ± 1.3	*n.a.*
Dihidroxiphenylpropan-2-ol	0.303±0.045	*n.a.*	*n.a.*
Cinnamic acids			
Caffeic acid	6.97±0.26	*n.a.*	*n.a.*
p-Coumaric acid	1.39±0.02	*n.a.*	*n.a.*
Ferulic acid	0.217±0.018	*n.a.*	*n.a.*
Coutaric acid	8.64±0.01	2.00 ± 0.12	*n.a.*
Caftaric acid	4.98±0.33	0.192 ± 0.071	*n.a.*
Stilbenes			
Resveratrol	7.12±0.29	0.427 ± 0.020	*n.a*
Resveratrol-3-Oglucoside	*n.a.*	9.17 ± 0.17	*n.a.*
Flavan-3-ols and others			
(+)-Catechin	51.6±1.7	9.90 ± 0.32	74.6 ± 0.09
(-)-Epicatechin	34.9±2.9	6.87 ± 0.15	67.7 ± 0.75
(-)-Epicatechin-3-O-gallate	*n.a.*	0.226 ± 0.018	26.2 ± 0.41
Procyanidin B1	79.1±0.9	11.1 ± 0.1	61.0 ± 1.42
Procyanidin B2	44.7±0.6	4.69 ± 0.10	45.1 ± 0.95
B2-3-O-gallate	*n.a.*	0.0271 ± 0.0106	1.80 ± 0.06
B2-3'-O-gallate	*n.a.*	0.0258 ± 0.0028	1.61 ± 0.01
Procyanidin B3	16.0±1.0	1.23 ± 0.02	20.4 ± 0.33
Procyanidin B4	12.9±0.3	0.827 ± 0.018	15.0 ± 0.13
Procyanidin B5	2.67±0.01	*n.a.*	*n.a.*
Procyanidin B7	5.75±0.15	*n.a.*	*n.a.*
Procyanidin C1	14.0±0.4	1.07 ± 0.04	7.07 ± 0.08
Other trimers	7.96±1.05	1.24 ± 0.09	6.81 ± 0.06 (t2)
Flavonols			
Quercetin	1.92±0.01	22.4 ± 0.6	*n.a.*
Myricetin	0.697±0.028	2.55 ± 0.07	*n.a.*
Kaempherol	*n.d.*	0.0366 ± 0.0055	*n.a.*
Quercetin-3-O-glucoside	*n.a.*	0.137 ± 0.023	*n.a.*
Quercetin-3-O-galactoside	*n.a.*	0.107 ± 0.006	*n.a.*
Anthocyanins			
Delphinidin-3-*O*-glucoside	2.58±0.11	0.568 ± 0.012	*n.a.*
Cyanidin-3-*O*-glucoside	0.761±0.041	0.265 ± 0.010	*n.a.*
Petunidin-3-*O*-glucoside	4.06±0.13	1.47 ± 0.03	*n.a.*
Peonidin-3-*O*-glucoside	18.9±2.0	1.78 ± 0.01	*n.a.*
Malvidin-3-*O*-glucoside	36.7±3.4	9.01 ± 0.50	*n.a.*

*n.d._not detected

*n.a._not analyzed

Table 2. Mean (±SD) of Log_{10}CFU values for *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, *V. dispar* after treatments with water, ethanol 12% in water, wine, dealcoholized wine and 0.2% clorhexidine-gluconate.

	S.mutans	*S. oralis*	*F.nucleatum*	*A.oris*	*V.dispar*
Water	8.09±0.09	8.42±0.17	5.90±0.89	8.40±0.32	7.36±0.37
Ethanol 12% in water	8.01±0.16	8.20±0.37	<1.30±0.00[a]	8.75±0.64	7.92±0.12
Wine	7.89±0.07	5.77±0.63[a]	<1.30±0.00[a]	8.37±0.20	6.94±0.38
Dealcoholized wine	7.68±0.22	4.79±0.80[a]	<1.30±0.00[a]	8.24±0.07	7.12±0.88
Clorhexidine-gluconate 0.2%	<1.30±0.00[a]	<1.30±0.00[a]	<1.30±0.00[a]	<1.30±0.00[a]	<1.30±0.00[a]

[a] Significant differences (Dunnett's test) in the population in comparison to the negative control (water).

Table 3. Mean (±SD) of Log_{10}CFU values for *S. mutans*, *S. oralis*, *A.oris*, *F. nucleatum*, *V. dispar* after treatments with Provinols™, Provinols™ + Vitaflavan®, Provinols™ + IDY 1 and Provinols™ + IDY 2.

	S.mutans	*S. oralis*	*F.nucleatum*	*A.oris*	*V.dispar*
Water + 2.5% DMSO	8.02±0.05	8.47±0.99	6.77±0.07	8.55±0.07	7.74±0.04
Provinols™	8.11±0.08	8.59±0.11	6.54±0.57	8.34±0.39	7.68±0.39
Provinols™ + Vitaflavan®	7.77±0.18	6.49±0.07[a]	<1.30±0.00[a]	<3.30±0.00[a]	7.95±0.09
Provinol™ + IDY 1	8.18±0.03	8.60±0.01	7.13±0.13	8.89±0.02	8.15±0.15
Provinols™ + IDY 2	8.13±0.07	8.44±0.07	7.14±0.04	8.68±0.03	8.11±0.11

[a] Significant differences (Dunnett's test) in the population in comparison to the negative control (water + 2.5% DMSO).

490

491 **FIGURE LEGENDS**

492 **Figure 1.** Biofilm formation/maturation and assays diagram

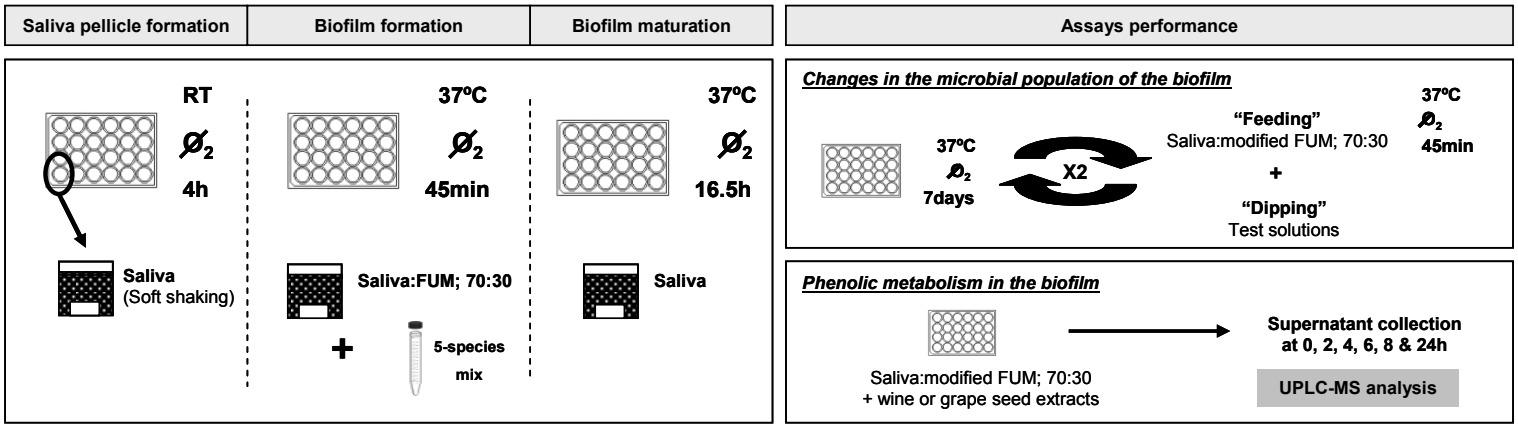
493 **Figure 2.** Confocal scanned segment of biofilm stained with LIVE/DEAD kit after exposure to
494 (A) negative control (water), (B) wine extract (Provinols™, 1.6 g/L), (C) red wine, and (D)
495 grape seed extract (Vitaflavan®, 2.5 g/L) in wine extract solution (1.6 mg/mL).

496 **Figure 3.** Metabolism of precursors (+)-Catechin, Quercetin and Procyanidine B2 after
497 0,2,4,6 8 and 24 hours of incubation in FUM media enriched with (A) Provinols™ and (B)
498 Vitaflavan® 1% in Provinols™ solution.

499

500 **FIGURES**

501



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503 **Figure 1.** Biofilm formation/maturation and assays diagram.

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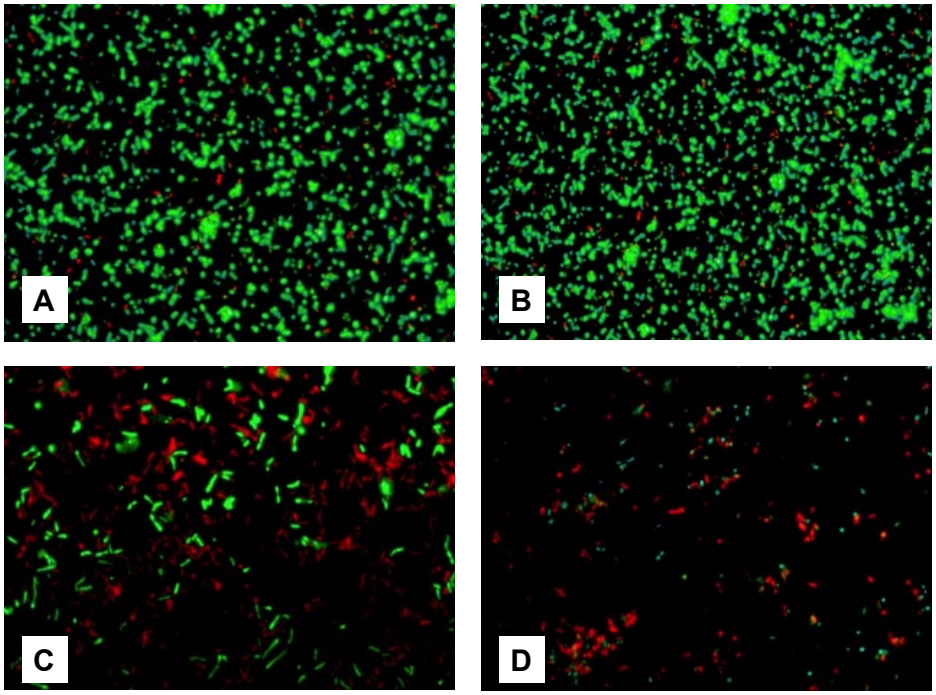

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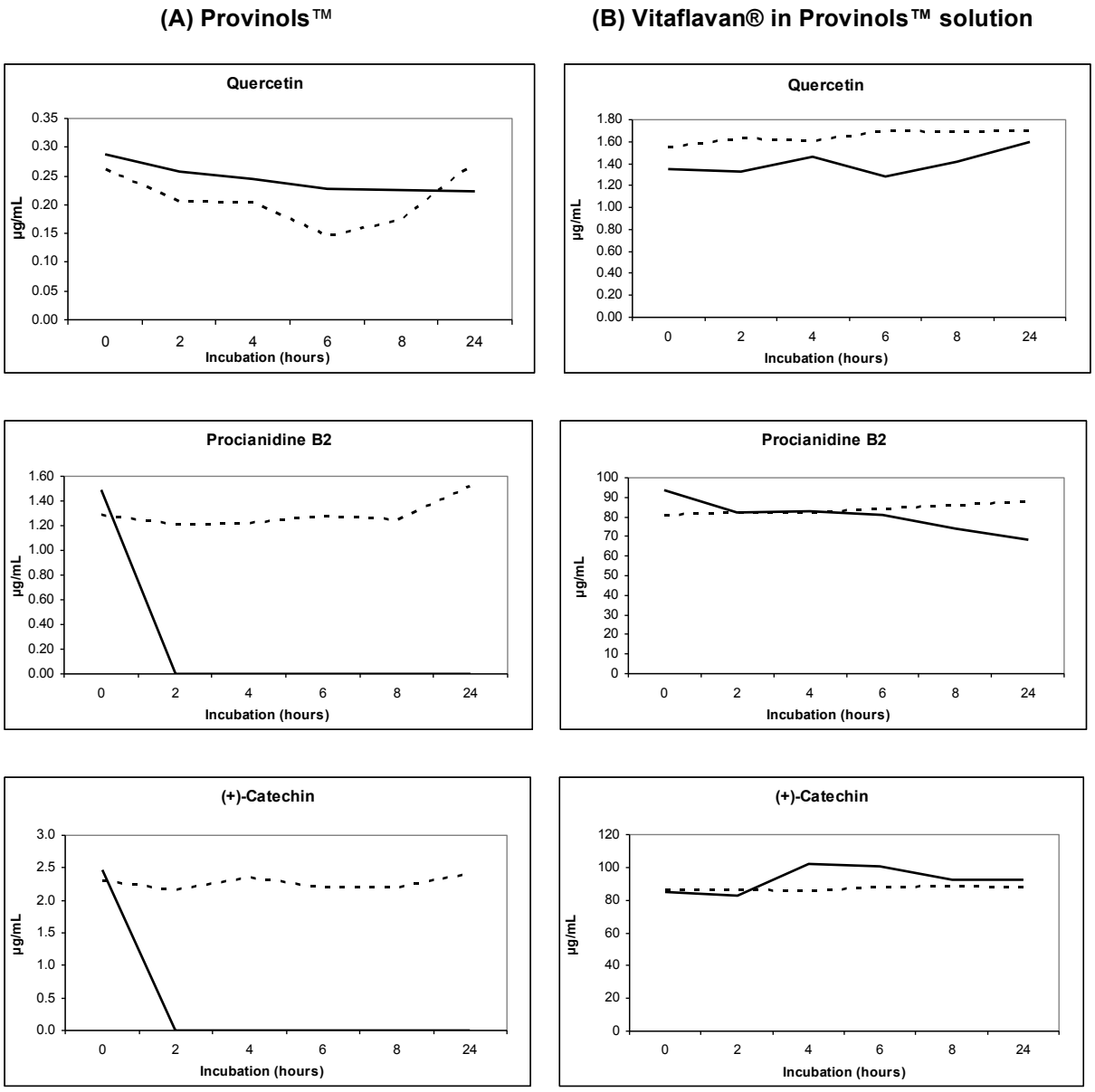

Figure 3. Metabolism of precursors (+)-Catechin, Quercetin and Procyanidine B2 after 0,2,4,6 8 and 24 hours of incubation in FUM media enriched with (A) Provinols™ and (B) Vitaflavan® 1% in Provinols™ solution.